REMARKS

Claims 89-117 are pending. Claims 89-110 and 114 are under examination. Claims 89 and 99 have been amended. Applicant has canceled claims 91, 93, 94, 102, 104 and 105 without prejudice to Applicant pursuing these claims in a related application. Support for the amendments to claims 89 and 99 can be found throughout the specification and the claims as filed. In particular, support for the amendment to claims 89 and 99 can be found on page 40, line 21 to page 42, line 3 and in originally filed claims 31.

Accordingly, these amendments do not raise an issue of new matter and entry thereof is respectfully requested.

Rejection Under 35 U.S.C. §103

Maintained Rejection

The rejection of claims 89-110 and 114 under 35 U.S.C. §103(a) as allegedly being obvious over Froesch et al., Proceeding of the American Association for Cancer Research, Annual Meeting, 89:13 (March 1998), in view of Takayama et al., Cancer Res. 58:3116-3131 (1998), Noordzij et al., J. Urology, 158:1880-1885 (1997), and Sano et al., U.S. Patent No. 5,665,539, is respectfully traversed. The Office has maintained the above rejection in view of the Declaration of Dr. Reed under 37 C.F.R. §1.132 submitted by the Applicant on July 1, 2009. To clarify the record, the Office Action states on page 4, lines 18-19 that the effective filing date of the instant claims is 7/7/2002. However, Applicant believes this is a typographical error based on the analysis provided on pages 2-4 of the Office Action, which concludes that the effective filing date of the instant claims is 07/07/2000. Nevertheless, Applicant respectfully maintains, for the reasons of record, that the claimed methods are unobvious over Froesch et al., alone or in combination with Takayama et al., Noordzij et al., and/or Sano et al.

Turning to the prior Office Action of May 29, 2008, which articulates the Office's maintained rejection, the Office asserts that Froesch et al. allegedly disclose BAG-1 protein (cytosolic BAG protein) is expressed in all 9/9 prostate cancer cell lines and 51/51 archival prostate tumor specimens and BAG-1L protein (nuclear BAG protein) is expressed in prostate

cancers and enhances androgen receptor function (see abstract and title). The Office also asserts that the general teachings of the art was that BAG-1 is anti-apoptotic and it prolongs cancer cell survival citing Yawata et al. Oncogene, 16:2681-2686 (1998); Tang et al., Journal of Clinical Oncology, 17(6):1710-1719 (1999); Yang et al., Exp. Cell Res., 247:200-207 (1999); and Takayama et al., *supra*. Therefore, it would have been allegedly *prima facia* obvious to one of ordinary skill in the art at the time of the invention and one would have been motivated to determine the level of BAG-1 expression in prostate cancer, compare the level with a reference level, and further correlate the level with the risk of tumor recurrence, tumor spread and survival in view of Froesch et al., Takayama et al. and the state of the art. The Office Action goes on to assert that one of ordinary skill would have allegedly had a reasonable expectation of success to do so because Froesch et al. had already successfully detected BAG-1 protein in all 9/9 prostate cancer cell lines and all 51/51 prostate tumor specimens, and Takayama et al. and the state of the art allegedly disclose that overexpression of BAG-1 protein promotes cell survival.

Claim 89, and all dependent claims thereof, are directed towards a method for determining the risk of tumor recurrence or spread in a patient suffering from prostate cancer by determining a BAG-1 gene expression level in a cancerous prostate tissue sample from the patient, wherein the BAG-1 gene expression level is determined by measuring cytosolic BAG-1 protein; and comparing the BAG-1 gene expression level in the patient to a reference BAG-1 gene expression level, the reference BAG-1 gene expression level being a level of BAG-1 gene expression above which correlates with an increased risk of tumor recurrence or spread and below which correlates with a decreased risk of tumor recurrence or spread, thereby determining the risk of tumor recurrence or spread in the patient. Claim 99, and all dependent claims thereof, are directed towards a method for determining a prognosis of survival in a patient suffering from prostate cancer by determining a BAG-1 gene expression level in a cancerous prostate tissue sample from the patient, wherein the BAG-1 gene expression level is determined by measuring cytosolic BAG-1 protein; and comparing the BAG-1 gene expression level in the patient to a reference BAG-1 gene expression level, the reference BAG-1 gene expression level being a level of BAG-1 gene expression above which correlates with decreased survival and below which correlates with increased survival, thereby determining a prognosis of survival in the patient.

Applicant respectfully maintains the position of record that none of Froesch et al., Takayama et al., Noordzij et al., or Sano et al., alone or in combination, render the claimed methods obvious. Although Applicant maintains the position of record, nevertheless to further prosecution, claims 89 and 99 have been amended to recite measuring cytosolic BAG-1 protein. With respect to Froesch et al., this reference, at best, describes BAG-1L expression in prostate cancer. Specifically, the title recites "BAG-1L protein is expressed in prostate cancers and enhances androgen receptor function." (emphasis added). Furthermore, the abstract recites that BAG-1L, not BAG-1, co-immunopreciptated with androgen receptors (AR) from LNCaP cell lysates and markedly enhanced the ability of androgen receptors to trans-activate reporter gene plasmids in PC3 and other cell lines. The abstract concludes by reciting "These findings implicate BAG-1L as a novel regulator of AR function in prostate cancers." (emphasis added). BAG-1L is the longer protein isoform of the BAG-1 gene, as described in the specification on page 10, line 22 to page 11, line 9 and is known to one of skill in the art to be the isoform which contains a nuclear localization signal in the N-terminal region of the protein (see Takayama et al. page 3121, Figure 3C). BAG-1L is described in the specification to be the isoform found in the nuclear portion of the cell (see page 40, lines 7-11). With respect to Takayama et al., this reference discloses on page 3130, right column, lines 8-22:

> In contrast, the BAG-1L protein was more variable in its expression, and in a few instances BAG-1L levels approached or were equivalent to the levels of ~36-kDa BAG-1 in tumor lines such as the breast cancer BT-549, the prostate cancer lines DU-145 and LN-CaP, and the leukemia line Jurkat. Indeed, breast cancer, prostate cancer, and leukemia cell lines were the most consistent expressors of the BAG-1L protein, with seven of seven prostate, seven of eight breast and four of five leukemia cell lines containing immunodetectable levels of this protein. An intriguing possibility is that BAG-1L, with its proclivity for nuclear targeting, may contribute to the regulation of nuclear hormone receptor function in these types of tumors, given the prominent role played by androgen receptor, ER, and glucocorticoid receptor in cancer of the prostate, breast and lymphoid organs, respectively. (emphasis added)

Takayama et al. also summarize in the last sentence of the abstract:

> In contrast to normal tissues, which only rarely expressed BAG-1L, tumor cell lines commonly contained BAG-1L protein,

> including most prostate, breast, and leukemia cell lines, suggesting that a change in BAG-1 mRNA translation frequently accompanies malignant transformation. (emphasis added)

Applicant respectfully submits that the claimed methods include the step of determining a BAG-1 gene expression level in a cancerous prostate tissue sample from the patient, wherein the BAG-1 gene expression level is determined by measuring cytosolic BAG-1 protein. In contrast, Froesch et al. provides no teaching or suggestion of determining BAG-1 expression for determining the risk of tumor recurrence or spread or prognosis for survival, let alone measuring cytosolic BAG-1 protein. Applicant respectfully submits that one of ordinary skill would have no expectation of success in using cytosolic BAG-1 protein levels to determine the risk of tumor recurrence or spread or determining a prognosis of survival of a prostate cancer patient based on the disclosure of Froesch et al., alone or in combination with Takayama et al., because BAG-1L protein was implicated as the novel regulator of AR function in prostate cancers as disclosed by Froesch et al and BAG-1L protein was the suggested isoform associated with malignant transformation as disclosed by Takayama et al. Neither reference, alone or in combination, teach or suggest detecting BAG-1 expression by measuring cytosolic BAG-1 protein or a correlation with risk of tumor recurrence or spread or a prognosis of survival for a prostate cancer patient.

Regarding Noordzij et al., Applicant maintains the arguments of record that Noordzij et al. found no correlation with Bcl-2. "The bcl-2 scores did not correlate with tumor stage or grade" (abstract; emphasis added). Noordzij et al. further indicated that "[A]ndrogen receptor scores were marginally related to tumor grade, but not to tumor stage" (abstract). Noordzij et al. stated that a "prognostic value of bcl-2 or androgen receptor in pretreatment transurethral resection specimens was not found" (see abstract and page 1883, right column, first complete paragraph). Noordzij et al. found only a combined bcl-2/androgen receptor score to be an independent prognostic marker to predict clinical progression (see abstract and page 1883, right column, third paragraph). Given the lack of prognostic value of bcl-2 as specifically stated in Noordzij et al., one skilled in the art would have had no reasonable expectation of success that determining BAG-1 gene expression level in cancerous prostate tissue, wherein the BAG-1 gene expression level is determined by measuring cytosolic BAG-1 protein, would allow determining the risk of tumor recurrence or spread or determining a prognosis of survival of a prostate cancer patient. Furthermore, Sano et al., at best, describes an immuno-PCT method. Therefore,

Applicant maintains that Noordzij et al. and/or Sano et al. cannot cure the deficiencies of Froesch et al. and/or Takayama et al.

Applicant respectfully maintains that Froesch et al., alone or in combination with Takayama et al., Noordzij et al. and/or Sano et al., does not teach or suggest the claimed methods. Absent such a teaching or suggestion, Applicant respectfully submits that the claimed methods are unobvious over Froesch et al., alone or in combination with Takayama et al., Noordzij et al. and/or Sano et al. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

New Rejection

The rejection of claims 89-110 and 114 under 35 U.S.C. §103(a) as allegedly being obvious over Froesch et al., Proceeding of the American Association for Cancer Research, Annual Meeting, 89:13 (March 1998), in view of Tang et al., Journal of Clinical Oncology, 17(6):1710-1719 (1999); Yawata et al., Oncogene, 16:2681-2686 (1998); and Sano et al., U.S. Patent No. 5,665,539, is respectfully traversed. As discussed above, the Office asserts that Froesch et al. allegedly disclose BAG-1 protein (cytosolic BAG protein) is expressed in all 9/9 prostate cancer cell lines and 51/51 archival prostate tumor specimens and BAG-1L protein (nuclear BAG protein) is expressed in prostate cancers and enhances androgen receptor function (see abstract and title). The Office asserts that Tang et al. allegedly disclose that BAG-1 is overexpressed in the majority of invasive breast carcinomas, and in multivariate analysis, BAG-1 expression was significantly associated with shorter disease-free and overall survival citing the Abstract and Figures 3 and 4. The Office asserts that Yawata et al. allegedly disclose that prolonged cell survival introduced by overproduction of BAG-1 strongly enhances peritoneal dissemination of human gastric cancer cells *in vivo* and that overexpression of BAG-1 leads to prolonged cell survival of murine melanoma B16 cells, and this enhanced anti-cell death activity promotes their pulmonary metastasis *in vivo*. The Office still further cites Sano et al. as allegedly disclosing detection of a protein using immuno-PCR. The Office asserts that one would have been motivated to determine the level of BAG-1 expression in prostate cancer using immuno-PCR, compare the level with a reference level and further correlate the results with the risk of tumor recurrence, tumor spread and survival in a patient suffering from prostate cancer

based on the disclosures of the cited reference. Furthermore, one of ordinary skill in the art would have allegedly had a reasonable expectation of success because Froesch et al. allegedly detected BAG-1 protein in all 9/9 prostate cancer cell lines and all 51-51 prostate tumor specimens, Tang et al. allegedly showed that BAG-1 is overexpressed in the majority of invasive breast carcinomas, and in multivariate analysis, BAG-1 expression was significantly associated with shorter disease-free and overall survival, and Yawanta allegedly showed that overexpression of BAG-1 increased the metastatic potential of tumor cells *in vivo*. Applicant respectfully submits that the claimed methods are unobvious over Froesch et al., alone or in combination with Tang et al., Yawata et al., and/or Sano et al.

As discussed above, Applicant respectfully submits that the disclosure of Froesch et al., at best, describes BAG-1L expression in prostate cancer. Furthermore, Applicant respectfully disagrees with the characterization of Tang et al. At best, Tang et al. appear to disclose that BAG-1 did not correlate with conventional prognostic factors and that nuclear expression of BAG-1 tended to be associated with a shorter disease free and overall survival in invasive breast cancer (see abstract and Figure 4), whereas the claimed methods include the step of determining a BAG-1 gene expression level in a cancerous prostate tissue sample from the patient, wherein the BAG-1 gene expression level is determined by measuring cytosolic BAG-1 protein. One of skill in the art would have no motivation to combine the description in Froesch et al. of the expression of BAG-1L in prostate cancer with the description of Tang et al. expression of nuclear expression of BAG-1 in breast cancer to achieve the claimed methods. Furthermore, even if combined, there would be no expectation of success because Tang et al. discloses that the nuclear expression of BAG-1 correlates with shorter disease free and overall survival (see abstract and Figure 4), whereas the claimed methods compare the patients cytosolic BAG-1 protein level to a reference BAG-1 gene expression level, wherein the reference BAG-1 gene expression level being a level of BAG-1 gene expression above which correlates with decreased survival and below which correlates with increased survival. Neither reference, alone or in combination, teach or suggest detecting BAG-1 expression by measuring cytosolic BAG-1 protein or a correlation with risk of tumor recurrence or spread or a prognosis of survival for a prostate cancer patient.

Regarding Yawata et al., Applicant submits that Yawata et al. at best disclose that overexpression of Bcl-2 or BAG-1 enhances peritoneal dissemination of human gastric MKN74 cells in nude mice (see Yawata at page 2682, left column, paragraph 1, lines 3-5; and page 2684 under the heading of *peritoneal dissemination of MKN74 transfectants*). One of skill in the art would have no motivation to combine the description in Froesch et al. of the expression of BAG-1L in prostate cancer, the description of Tang et al. of the expression of nuclear expression of BAG-1 in breast cancer and the description of Yawata et al. of the metastatic potential of gastric cancer cells overexpressing BAG-1 to achieve the claimed methods. For a person having ordinary skill in the art and having the capability of appreciating the complexity of scientific issues in cancer, the BAG-1 effects in dissemination of gastric cancer cells in mice would not have provided any reasonable expectation of success for determining the risk of tumor recurrence or spread in patients suffering from prostate cancer or determining a prognosis of survival of a prostate cancer patient, as claimed. Furthermore, Sano et al., at best, describes an immuno-PCT method. Therefore, Applicant submits that Yawata et al. and/or Sano et al. cannot cure the deficiencies of Froesch et al. and/or Tang et al.

Applicant respectfully submits that Froesch et al., alone or in combination with Tang et al., Yawata et al. and/or Sano et al., does not teach or suggest the claimed methods. Absent such a teaching or suggestion, Applicant respectfully submits that the claimed methods are unobvious over Froesch et al., alone or in combination with Tang et al., Yawata et al. and/or Sano et al. Accordingly, Applicant respectfully requests that this rejection be withdrawn.

CONCLUSION

In light of the amendments and remarks herein, Applicant submits that the claims are now in condition for allowance and respectfully requests a notice to this effect. The Examiner is invited to call the undersigned agent if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

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EXHIBIT 1

Oncogene (1998) 16, 2681–2686

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SHORT REPORT

Prolonged cell survival enhances peritoneal dissemination of gastric cancer cells

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Bcl-2 and a Bcl-2-binding protein BAG-1 function in protection from apoptosis induced by a variety of stimuli. Deregulated expression of Bcl-2 leads to inhibition of apoptosis and is correlated with development of various cancers. Here, we provide evidence that prolonged cell survival introduced by overproduction of Bcl-2 or BAG-1 strongly enhances peritoneal dissemination of human gastric cancer MKN74 cells. Gene transfer-mediated overexpression of Bcl-2 or BAG-1 led to prolonged cell survival of MKN74 cells against serum-starved apoptosis and anoikis. When the viable transfectants were inoculated into the intraperitoneal cavity of BALB/c nude mice, the Bcl-2-expressing MKN74 cells and the BAG-1-expressing MKN74 cells exhibited strongly enhanced peritoneal dissemination in BALB/c nude mice and whole disseminated tumor weights were increased by 4-fold and 3.3-fold, respectively, compared with the control transfectants. The enhanced peritoneal dissemination of MKN74-Bcl-2 and MKN74-BAG-1 transfectants correlated well with resistance to cell death induced by serum-starvation and anoikis. However, the overexpression of Bcl-2 or BAG-1 caused no significant difference among the transfectants in cell growth rates, either *in vitro* or *in vivo*. Taken together, these studies demonstrate that resistance to apoptosis is a crucial factor for development of peritoneal dissemination of human gastric cancer cells.

Keywords: BCL-2; BAG-1; gastric adenocarcinoma; peritoneal dissemination

Apoptosis plays a crucial role in the regulation of embryogenesis, organ development, immune system, repertoire selection and tissue homeostasis. Since apoptosis is responsible for removal of cells that are no longer necessary or dangerous to the host, disturbance of physiological apoptosis may lead to the abnormal cell growth and thus is likely to be associated with tumor progression in a variety of malignancies. Bcl-2 is one of the key molecules for inhibiting apoptosis (Reed, 1993). It has been demonstrated that Bcl-2 is aberrantly expressed in a wide variety of malignant tumors, such as lung and prostate cancers, follicular lymphoma and chronic lymphocytic leukemia (Pezzella *et al.*, 1993; McDonnell *et al.*, 1992; Tsujimoto and Croce 1986; Adachi *et al.*, 1990). These findings strongly suggest that aberrant Bcl-2 expression may be an important factor for tumorgenesis. Several Bcl-2 homolog and Bcl-2-binding proteins have been identified which participate in either protection from or promotion of apoptosis. Bcl-2 family proteins share at least one of several conserved regions BH1 to BH4. Many but not all Bcl-2 family proteins can interact with themselves or other members of the family (Zha *et al.*, 1996). A Bcl-2-binding protein BAG-1 has been identified. BAG-1 prolongs cell survival in concert with Bcl-2 (Takayama *et al.*, 1995). Since BAG-1 has no conserved regions BH1 to BH4, it is not a member of the Bcl-2 family and may have a unique function in protection from apoptosis.

Peritoneal dissemination is the most common form of recurrence after surgical total resection of many primary cancers, such as gastric, ovarian and pancreatic adenocarcinoma. Thus, the prevention and treatment for peritoneal dissemination are crucial therapeutic targets for these cancers. To prevent this type of recurrence, several efforts have been made. For example, intra-abdominal administration of anti-cancer drugs and abdominal hyperthermia. However, peritoneal dissemination of these cancer cells still remains the most formidable obstacles in their therapy. Although reduction of peritoneal dissemination is essential to improve postoperative survival, few studies have been reported about the process of peritoneal dissemination.

During the process of peritoneal dissemination, gastric cancer cells detach from their primary locations and thereafter survive without adequate interactions between their surface molecules and extracellular matrix proteins. Apoptosis caused by detachment of epithelial cells has been termed 'anoikis' (Frisch and Francis, 1994). Since overexpression of Bcl-2 is known to protect epithelial cells against anoikis, levels of Bcl-2 expression may be a crucial factor for peritoneal dissemination of gastric cancer cells. Though BAG-1 was first discovered because of the ability to associate with Bcl-2, recent study reveals that BAG-1 can also physically associate with hepatocyte growth factor receptors (HGFR) and enhance transduction of HGFR-mediated signals that promote cell survival. Since HGFR is highly expressed in most gastric cancers (Bardelli *et al.*, 1996; DI Renzo *et al.*, 1991), the interaction of BAG-1 with HGFR may be relevant to the progression of these tumors. These accumulating data strongly suggest that overexpression of Bcl-2 or BAG-1 may affect metastasis. In this regard, we have demonstrated previously that overexpression of Bcl-2 or BAG-1 leads to prolonged cell survival of murine

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melanoma B16 cells and this enhanced anti-cell death activity promotes their pulmonary metastasis (Takaoka *et al.*, 1997). We report here that overexpression of Bcl-2 or BAG-1 enhances peritoneal dissemination of human gastric cancer MKN74 cells. This increased dissemination potential is associated neither with altered cell morphology nor with enhanced cellular growth, but is well correlated with the ability of Bcl-2 or BAG-1 to protect these cells from anoikis. Thus, our data indicate a linkage of prolonged survival of gastric cancer cells in the absence of cell attachment to extracellular matrix proteins with their ability to disseminate intraperitoneally.

Expression of Bcl-2 and BAG-1 in human cancer cell lines

Overexpression of Bcl-2 is predominantly detected in early-stage rather than advanced-stage gastric adenocarcinomas (Bronner *et al.*, 1995). BAG-1 is ubiquitously expressed in a variety of normal cell types (Takayama *et al.*, 1995), but its expression in gastric cancer has not been explored. We thus investigated expression of Bcl-2 and BAG-1 proteins in various human adenocarcinoma cell lines. Human gastric cancer MKN45 cells contained relatively high levels of Bcl-2 proteins (Figure 1a). BAG-1 was clearly detectable in all cell lines examined, with MKN45 cells showing the highest expression levels (Figure 1a). Thus, cooperative expression of Bcl-2 and BAG-1 is observed in a gastric cancer cell line MKN45, which is consistent with the previous data showing the cooperative *bcl*-2 and *bag*-1 mRNAs expression in hematopoietic cells (Adachi *et al.*, 1996).

Increased apoptosis resistance of MKN74 cells expressing Bcl-2 or BAG-1

The well-differentiated gastric cancer cell line MKN74 expressed the lowest expression levels of Bcl-2 and

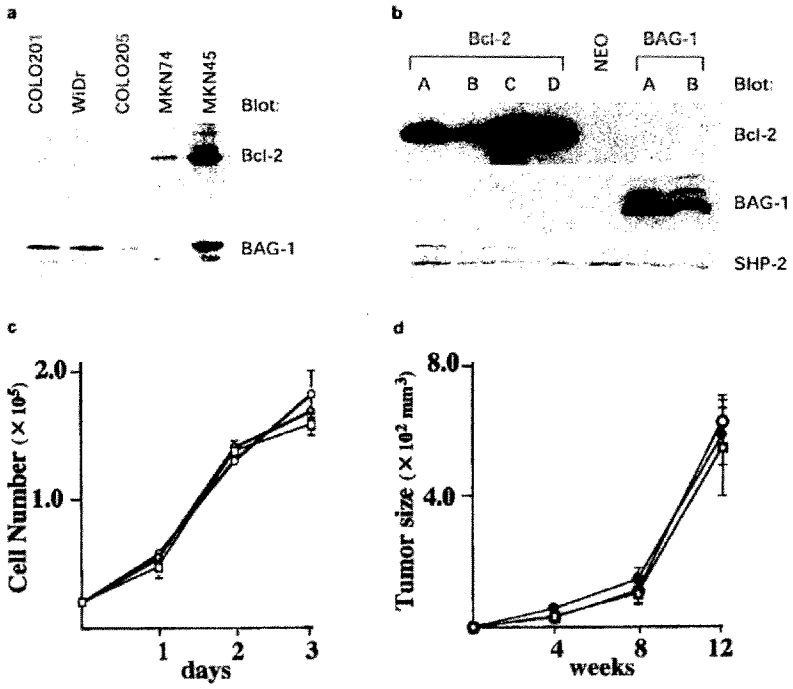

Figure 1 Immunoblot analysis and cell growth of Bcl-2-expressing or BAG-1-expressing MKN74 cells. (a) Cell lysates (50 μg/lane) from various human cancer cell lines were subjected to SDS–PAGE/immunoblotting using an antibody specific for Bcl-2 (DAKO) or BAG-1 as described previously (Takaoka *et al.*, 1997). Human gastric cancer cell lines MKN74 and MKN45, as well as human colon cancer cell lines COLO201, WiDr and COLO205 cells were provided from Japanese Cancer Research Resources Bank Corp (Tokyo, Japan). MKN45 and MKN74 cells were from poorly differentiated and well-differentiated types of gastric cancers, respectively (Asao *et al.*, 1994). The blots were developed by a standard enhanced chemiluminescence (ECL) method (Amersham). (b) Cell lysates (50 μg/lane) from the MKN74 cells transfected with *bcl*-2, *bag*-1 cDNA or the pcDNA3 plasmid alone (NEO) were subjected to SDS–PAGE/immunoblotting with anti-Bcl-2 or anti-BAG-1 antibodies. The full-length human *bcl*-2 cDNA pB4 (Tsujimoto and Croce, 1986), kindly obtained from Dr Y Tsujimoto (Osaka University, Japan), and the full-length murine *bag*-1 cDNA (Takayama *et al.*, 1995) were cloned into the eukaryotic expression pcDNA3 vector (Invitrogen) and were transfected into MKN74 cells using Lipofection technique (BRL). The filter was stripped and reprobed with anti-SHP-2 antibody to verify loading of equal amounts of protein. (c) *In vitro* cell growth of the transfectants; 0.25×10^5 viable transfectants were cultured for the indicated times. The culture cells were trypsinized and the viable cell numbers were counted. (d) *In vivo* growth rates of the transfectants; 10^6 viable transfectants in 0.5 ml Hank's balanced salt solution (HBSS; GIBCO BRL) were inoculated subcutaneously into BALB/c nude mice ($n = 5$). Tumor sizes were measured as their calculated volumes $[(1/2) \times (\text{longest diameter}) \times (\text{shortest diameter})^2]$ for the weeks indicated. Data represented the mean ± s.d. of five independent experiments of MKN74-Bcl-2 (circle), MKN74-BAG-1 (diamond) and MKN74-NEO (square). Using the Student's *t*-test, no significant differences were seen among the transfectants for both *in vitro* and *in vivo* cell growth rates

BAG-1 (Figure 1a) therefore was employed to investigate the effect of overexpression of Bcl-2 or BAG-1. For these experiments, *bcl*-2 and *bag*-1 cDNAs encoding either full-length Bcl-2 (Tsujimoto and Croce, 1986) or BAG-1 (Takayama *et al*, 1995) proteins were stably introduced into MKN74 cells. These gastric cancer cells are capable of dissemination in the murine peritoneal cavity (Asao *et al*., 1994). After selection in neomycin, the Bcl-2 or BAG-1 transfectant cells were analysed by immunoblotting using Bcl-2- and BAG-1-specific antibodies. Elevated levels of Bcl-2 protein were detected in the MKN74-Bcl-2 transfectants but not in the MKN74-NEO transfectants that received the pcDNA3 parental control plasmid alone (Figure 1b). Although BAG-1 expression was detectable in MKN74 parental cells, markedly elevated levels of BAG-1 protein were found in the MKN74-BAG-1 transfectants as compared with the MKN74-NEO transfectants (Figure 1b). We chose the MKN74-Bcl-2 (clone A) and the MKN74-BAG-1 (clone A) as representative transfectants overexpressing either Bcl-2 and BAG-1, respectively.

We explored the ability of Bcl-2 or BAG-1 to drive cell proliferation in MKN74 cells. When the transfectants were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), their growth rates were not significantly different (Figure 1c). Thus, overexpression of neither Bcl-2 nor BAG-1 proteins affected proliferation of MKN74 cells *in vitro*. In addition, the transfectants (10^6 cells per mouse) were inoculated subcutaneously into nude mice and their tumor sizes were measured. Similar size tumors were produced by all transfected cell lines (Figure 1d). Thus, overexpression of Bcl-2 or BAG-1 protein did not affect cellular growth of MKN74 cells, either *in vivo* or *in vitro*.

We next analysed the relative sensitivity of the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants to apoptosis induced by serum-starvation. At 2 days after serum starvation, loss of cell viability (% of total cells) in the MKN74-NEO, MKN74-BAG-1 and MKN74-Bcl-2 was $59 \pm 5.1\%$, $33 \pm 2.4\%$ and $29 \pm 5.8\%$, respectively (Figure 2a). Thus, overexpression of Bcl-2 or BAG-1 led to resistance to cell death induced by serum starvation. We further examined their DNA fragmentation reflecting cleavage of internucleosomal sites, which is generally associated with the apoptotic process. DNA from the MKN74-NEO transfectants cultured without FBS for 2 days exhibited significant amounts of fragmentation (Figure 2b). In contrast, far less DNA fragmentation was observed in serum-starved cultures of the MKN74-Bcl-2 and MKN74-BAG-1 transfectants (Figure 2b). From these experiments, overexpression of Bcl-2 or BAG-1 in MKN74 cells increased resistance to apoptosis induced by serum starvation.

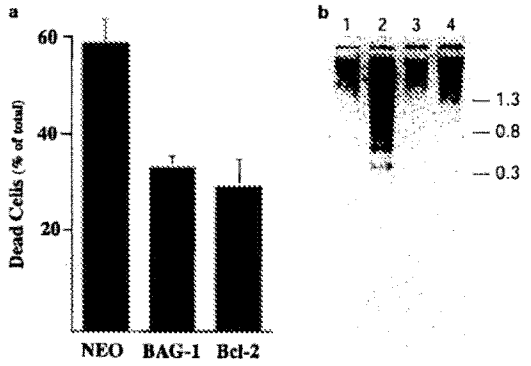

Figure 2 Resistance of transfectants against serum starvation-induced apoptosis. (**a**) Superior resistance to serum starvation in the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants; transfectants were cultured for 2 days at 5×10^5 cells/ml without FBS. Dead cells were assessed by the loss of ability of the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants to exclude trypan blue. Data represented the mean $\pm$ s.d. of three independent experiments. (**b**) DNA fragmentation in the serum-starved MKN74 transfectants. The transfectants were seeded at 4×10^6 cells per 100 mm plate without FBS and cultured for 2 days. After washing with PBS, their low molecular weight genomic DNA was extracted with 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris, pH 7.4 at 4°C for 10 min. The DNA was treated with 400 μg/ml of RNase A for 1 h at 37°C, incubated with 400 μg/ml of Proteinase K for 1 h at 37°C, ethanol precipitated and analysed (Frisch and Francis, 1994). DNAs from the proliferating MKN74-NEO (lane 1), the starved MKN74-NEO (lane 2), the starved MKN74-BAG-1 (lane 3) and the starved MKN74-Bcl-2 (lane 4) transfectants were loaded into 1.2% agarose gels which were stained with 1 μg/ml of ethidium bromide and photographed under U.V. light. The size markers to the right were from ΦX174 DNA/*Hae*III digested DNA (kb)

Bcl-2 and BAG-1 increase resistance to anoikis

After inoculation into the murine peritoneal cavity, cancer cells must survive without adequate cell-matrix or cell-cell interactions. We thus examined the survival of the transfectants under anoikis conditions. The transfectants were counted and plated onto 100 mm petri-dishes, which had been coated with polyHEMA. They could no longer attach the plate and their viability gradually reduced after 6 h culture under anoikis condition (Figure 3a). However, overexpression of Bcl-2 or BAG-1 clearly reduced loss of cell viability. When 24 h culture under anoikis condition, the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants exhibited prolonged cell survival compared with the MKN74-NEO transfectants (approximately twofold and 1.6-fold increase, respectively) (Figure 3), suggesting that these transfectants had an enhanced ability to survive without adequate cell-matrix interactions. The prolonged cell survival of unattached cells substantially reduced DNA fragmentation in the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants compared with the MKN74-NEO transfectants (Figure 3c). To confirm whether the loss of cell viability was due to anoikis, we analysed the effect of β1 integrin (CD29)-mediated signaling using anti-β1 antibody K20. The anti-β1 antibody K20 stimulates tyrosine phosphorylation of proteins of 115 to 130 kDa in an epidermal carcinoma cell line (Kornberg *et al*., 1991). Since this signal mimics the attachment of the cells via β1 integrin to fibronectin, anti-β1 integrin antibody-mediated stimulation may suppress anoikis. In all MKN74 transfectants, addition of K20 greatly suppressed loss of cell viability induced by detachment, allowing their cell death to reduce approximately 60% of control transfectants (Figure 3b). This implies that the loss of cell viability induced by detachment can be rescued by integrin β1-mediated signaling, though its effect is

partial, indicating that their cell death is due to anoikis. Since the inhibitory effects of K20 against their anoikis showed no significant difference among the transfectants (Figure 3b), Bcl-2 or BAG-1-mediated anti-anoikis activity seems not to be directly associated with integrin β1-mediated signaling. However, it is still possible that overexpression of Bcl-2 or BAG-1 may enhance β1 integrin-mediated signaling though unlikely.

Peritoneal dissemination of MKN74 transfectants

To explore the correlation of anti-cell death activity with peritoneal dissemination of MKN74 cells, the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants were inoculated into abdominal cavities of nude mice. Six weeks after the inoculation, the MKN74-Bcl-2 and MKN74-BAG-1 transfectants produced more extensive peritoneal metastatic lesions than the MKN74-NEO transfectants. Metastatic lesions exhibited extensive involvement of moderately differentiated adenocarcinoma from MKN74 transfectants, and histological sections of a representative tumor from the MKN74-Bcl-2 transfectants are shown in Figure 4a and b. There was no significant difference of pathological findings among the transfectants (data not shown). We next evaluated their dissemination potential by comparison of the whole tumor weights in the murine peritoneal cavity. The MKN74-Bcl-2 and MKN74-BAG-1 transfectants showed clearly elevated peritoneal dissemination by fourfold (4.0 ± 0.5) and 3.3-fold (3.3 ± 0.6) higher weights, respectively compared with the MKN74-NEO transfectants (Figure 4c). Other experiments using other independently derived MKN74-Bcl-2 and MKN74-BAG-1 over-expressing cell lines produced similar results; the MKN74-Bcl-2 transfectants (clone C in Figure 1b) and MKN74-BAG-1 transfectants (clone B) showed clearly elevated peritoneal dissemination with fourfold (4.0 ± 0.6) and 3.6-fold (3.6 ± 0.5) higher weights, compared with the MKN74-NEO transfectants (data not shown).

In the present data, we provide evidence that gene transfer-mediated overexpression of Bcl-2 or BAG-1 promotes peritoneal dissemination of a human gastric cancer cell line MKN74. Theoretically, this promotion of peritoneal dissemination of gastric cancer cells might be caused by biological activities related to overexpression of Bcl-2 or BAG-1 protein other than their anti-cell death activity. However, these transfectants exhibited similar growth rates and produced subcutaneous tumors with similar sizes compared to MKN74-NEO control cells. In addition, their cellular morphology was not significantly different. We thus exclude possibilities that the promotion of peritoneal dissemination by overexpression of Bcl-2 or BAG-1 proteins was due to the enhanced proliferation or altered expression of cell surface molecules may affect cell-cell interactions. Overexpression of two different apoptosis antagonists Bcl-2 and BAG-1 similarly enhanced peritoneal dissemination (Figure 4), and MKN45 cells expressing high levels of Bcl-2 and BAG-1 (Figure 1) exhibited significantly larger amount of peritoneal dissemination in nude mice than MKN74 cells (Asao *et al.*, 1995). These data suggest that prolonged cell survival can promote peritoneal dissemination of MKN74 cells. When combined with our previous data demonstrating that overexpression of Bcl-2 or BAG-1 in murine malignant melanoma cells leads to enhanced pulmonary metastasis (Takaoka *et al.*, 1997), our data strongly suggest that resistance to apoptosis is a crucial factor for tumor cell metastasis in a wide variety of malignancies.

To accomplish peritoneal dissemination, cancer cells must survive without cell-cell interactions or cell-matrix

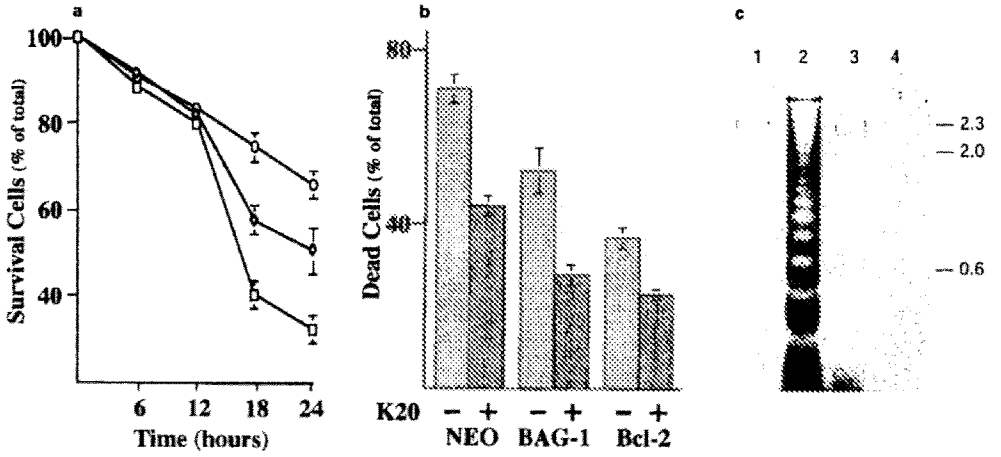

Figure 3 Resistance of Bcl-2 and BAG-1 transfectants against anoikis. (a) Transfectants were cultured at 5×10^5 cells/ml in polyHEMA (Aldrich Chemical Co., Milwaukee, WI) coated plates for the indicated times. Cell survival was assessed by trypan blue exclusion assay, for the MKN74-Bcl-2 (circle), MKN74-BAG-1 (diamond) and MKN74-NEO (square) transfectants. (b) Transfectants were cultured at 5×10^5 cells/ml in polyHEMA coated plates for 24 h with (+) or without (−) addition of anti-integrin β1 antibody K20 (10 μg/ml; Coulter) and polyclonal rabbit anti-mouse antibody (20 μg/ml; Jackson Laboratories). Dead cells were assessed by failure to exclude trypan blue. Data represented the mean ± s.d. of three independent experiments. (c) DNA fragmentation in the transfectants under anoikis condition. The control MKN74-NEO (lane 1), and the MKN74-NEO (lane 2), MKN74-BAG-1 (lane 3) and MKN74-Bcl-2 (lane 4) transfectants were cultured under anoikis conditions for 24 h and their germline DNAs were loaded into 1.2% agarose gels. The size markers to the right were from λ phage *Hind*III-digested DNA (kb)

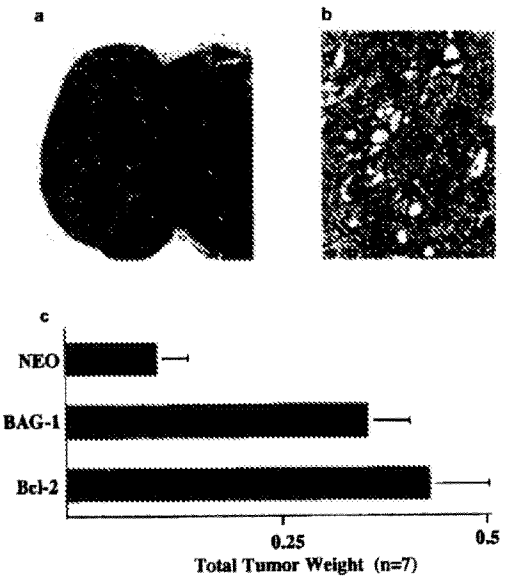

Figure 4 Peritoneal dissemination potential of MKN74-transfectants. (**a**) Representative whole mount specimen of a tumor in an abdominal cavity where the MKN74-Bcl-2 transfectants were inoculated (original magnification × 24). The tumors were preserved in neutralized 10% formaldehyde solution, subsequently followed by pathological investigation. (**b**) All tumors were predominantly composed of moderately-differentiated adenocarcinoma (original magnification × 240). (**c**) Abdominal dissemination potential of the MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants was evaluated by increases of tumor weights in the abdominal cavity. Extensive peritoneal metastatic lesions were found in mice that the MKN74-Bcl-2 or MKN74-BAG-1 transfectants were inoculated, whereas far less metastatic lesions were found in mice the MKN74-NEO transfectants were inoculated. The viable MKN74-Bcl-2, MKN74-BAG-1 and MKN74-NEO transfectants (10^6 cells) in 0.5 ml HBSS were injected into the abdominal cavity of nude mice ($n=7$). Six weeks after the inoculation of the transfectants, the mice were sacrificed and examined whether they developed peritoneal dissemination. Bars represent the means ± s.d. of whole tumor weights in grams ($n=7$ per each transfectant)

interactions prior to adhesion of cancer cells to the murine peritoneal mesothelium. Although several studies indicate that loss of requirements for cell-cell interactions in gastric cancers promotes tumor progression (Oda *et al*., 1994), we could not find any significant difference in cell aggregation among transfectants (data not shown). To explore possible mechanisms for linking anti-cell death activity and enhanced peritoneal dissemination of the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants, we investigated their cell survival under anoikis conditions. It has been reported that after the inoculation of cancer cells into peritoneal cavity, the first adhesion of cancer cells to the peritoneum takes place on days 5–7, and the cancer cells begin to proliferate and infiltrate the muscle layer on days 9–11 (Buck *et al*., 1973). This suggests that the MKN74 transfectants must survive for at least 5 days without adequate cell-matrix interactions before they successfully invade the murine peritoneum. Our anoikis assays strongly suggest that the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants exhibited superior viability under anoikis conditions, in comparison with control transfectants. This implies that the MKN74-Bcl-2 and the MKN74-BAG-1 transfectants can survive for a longer period in the peritoneal cavity than the MKN74-NEO transfectants. The prolonged cell survival under anoikis conditions was well correlated with enhanced peritoneal dissemination of the transfectants (Figures 3 and 4). Thus, it is likely that the MKN74-Bcl-2 and MKN74-BAG-1 transfectants gain enhanced peritoneal dissemination potential by reduced requirement of cell-matrix interaction via prolonged cell survival.

It has been shown that overexpression of Bcl-2 strongly inhibits apoptosis induced by disruption of epithelial cell-matrix interactions (Frisch and Francis, 1994), and that interactions with extracellular matrix suppress apoptosis in mammary epithelial cells (Boudreau *et al*., 1995). Here, our data clearly demonstrate that overexpression of BAG-1 inhibits anoikis of epithelial cells, in addition to Bcl-2. It is of interest to investigate whether anti-apoptotic molecules generally can inhibit anoikis and whether Bcl-2 or BAG-1 is functionally associated with integrin $\beta 1$-mediated signaling.

Several studies have examined the levels of Bcl-2 expression in normal gastric epithelial cells and gastric cancers. Bcl-2 protein is highly expressed in the earliest precursor dysplastic lesions of gastrointestinal epithelium, as well as majority of gastric adenocarcinomas (Bronner *et al*., 1995). Immunohistochemical studies have failed to demonstrate a correlation between Bcl-2 expression and overall patient survival (Lauwers *et al*., 1995). These findings imply that the detection of Bcl-2 protein alone is not likely to reflect anti-apoptotic activity in the cancer cells, since many other molecules, which function as either inhibitors or promoters of apoptosis, may also play crucial roles in the tumor cells and cooperatively determine their fate. In this context, little information is available that links anti-apoptotic activity to metastatic potential or peritoneal dissemination of gastric cancers. Thus, the data indicating enhancement of peritoneal dissemination of gastric cancer cells by overexpression of either Bcl-2 or BAG-1 proteins reveals a crucial role of anti-apoptotic activity during development of peritoneal dissemination of gastric cancer cells. If we find means which can counteract the effects of anti-apoptotic proteins such as Bcl-2 and BAG-1, these therapies may greatly improve suppression of tumor metastasis.

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